

DEVELOPMENT AND DISEASE

Bone marrow-derived cells as progenitors of lung alveolar epithelium

Darrell N. Kotton*, Bei Yang Ma, Wellington V. Cardoso, Elisabeth A. Sanderson, Ross S. Summer, Mary C. Williams and Alan Fine

The Pulmonary Center, Boston University School of Medicine, 715 Albany Street, Boston, Massachusetts 02118, USA

*Author for correspondence (e-mail: dkotton@yahoo.com)

Accepted 5 October 2001

SUMMARY

We assessed the capacity of plastic-adherent cultured bone marrow cells to serve as precursors of differentiated parenchymal cells of the lung. By intravenously delivering *lacZ*-labeled cells into wild-type recipient mice after bleomycin-induced lung injury, we detected marrow-derived cells engrafted in recipient lung parenchyma as cells with the morphological and molecular phenotype of type I pneumocytes of the alveolar epithelium. At no time after marrow cell injection, did we detect any engraftment as type II pneumocytes. In addition, we found that cultured

and fresh aspirates of bone marrow cells can express the type I pneumocyte markers, T1 α and aquaporin-5. These observations challenge the current belief that adult alveolar type I epithelial cells invariably arise from local precursor cells and raise the possibility of using injected marrow-derived cells for therapy of lung diseases characterized by extensive alveolar damage.

Key words: Alveolar epithelium, Bone marrow, Stem cells, Type I pneumocytes

INTRODUCTION

Accumulating data demonstrate that bone marrow-derived cells can serve as precursors for differentiated cells of multiple organs. In these studies, adult bone marrow cells give rise to tissue structures previously held to be derived only from one of the original embryonic cell layers (Eglitis and Mezey, 1997; Ferrari et al., 1998; Gussoni et al., 1999; Kopen et al., 1999; Lagasse et al., 2000; Orlic et al., 2001; Pereira et al., 1995; Pereira et al., 1998; Petersen et al., 1999; Prockop, 1997; Woodbury et al., 2000). This has challenged long held views regarding the potential origin of somatic stem cells and the fixed nature of stem cell repertoire. Based on *ex vivo* experimental assays of unlimited self-renewal and clonogenicity, subtypes of bone marrow-derived cells meet the classical criteria for stem cells (Bianco and Robey, 2000; Lemischka, 1999; Prockop, 1997). In addition, but no less important, progenitor cells with more limited proliferative and differentiation repertoire also exist in the bone marrow (Bianco and Robey, 2000; Lemischka, 1999; Prockop, 1997).

To date, systemically injected mouse bone marrow-derived cells have been demonstrated to differentiate into parenchymal cells of various non-hematopoietic tissues including muscle, cartilage, bone, liver, heart, brain, intestine and lung (Alison et al., 2000; Eglitis and Mezey, 1997; Ferrari et al., 1998; Gussoni et al., 1999; Hou et al., 1999; Kopen et al., 1999; Krause et al., 2001; Lagasse et al., 2000; Nilsson et al., 1999; Orlic et al., 2001; Petersen et al., 1999; Prockop et al., 2000; Woodbury et al., 2000). The engrafted marrow cells are able to adopt the morphologic and molecular phenotype of their new resident

organ. In the lung, bone marrow-derived cells have been detected in recipient lung tissue as fibroblast-type cells (Ding et al., 1999; Ono et al., 1999; Pereira et al., 1995; Pereira et al., 1998) and recently as differentiated bronchial epithelium and alveolar type II pneumocytes, raising the possibility of utilizing marrow to generate lung epithelium (Krause et al., 2001). The easy accessibility of bone marrow cells, along with their ability to adopt new phenotypes, has broad implications for disease therapy of many organ systems.

Fresh bone marrow cultured in plastic dishes can be separated into two general populations (Bianco and Robey, 2000; Phinney et al., 1999; Prockop, 1997). Cells that remain floating in the culture dish supernatant media are classical hematopoietic stem cells. Cells that adhere to the plastic culture dish grow into heterogeneous colonies of three discernible morphologic subtypes: small rounded cells positive for factor VIII antigen and CD-31 that may be of endothelial stem cell lineage, small stellate cells positive for mac-1/CD11b and CD-45 that are likely of myeloid lineage, and large polygonal fibroblast-like cells that synthesize matrix proteins such as collagen I, collagen IV, laminin and fibronectin (Conget and Minguell, 1999; Pittenger et al., 1999; Prockop, 1997). This latter cell type can serve as precursor for bone, muscle, fat and cartilage tissue (Bianco and Robey, 2000; Hou et al., 1999; Mackay et al., 1998; Nilsson et al., 1999; Phinney et al., 1999; Pittenger et al., 1999; Prockop, 1997), and can, under certain experimental conditions, transdifferentiate into non-mesodermal cell types (Kopen et al., 1999; Liechty et al., 2000; Prockop et al., 2000; Woodbury et al., 2000). A notable example of transdifferentiation is the adoption of a neural cell

morphology and molecular phenotype during culturing (Woodbury et al., 2000) and following direct injection of these cells into the brain (Kopen et al., 1999).

We have explored the fate and phenotype of cultured plastic-adherent bone marrow cells that engraft in lung tissue. Our results demonstrate that engrafted cells can acquire the morphological and molecular features of type I cells of the alveolar epithelium. While limited engraftment occurs in the normal lung, we found that engraftment is facilitated after bleomycin-induced lung injury. These novel findings extend the list of differentiated cell types that bone marrow can adopt in vivo. In addition, they provide a new experimental model for characterizing the molecular signals that control lung epithelial cell differentiation.

MATERIALS AND METHODS

Mice

Donor bone marrow was harvested from 1 month old B6,129S GTRosa26 or C57BL/6 GTRosa26 female mice. These mice constitutively express the *lacZ*/neomycin resistance transgene (*lacZ*/neo) in all tissues. *lacZ* expression is detectable after X-gal staining, which imparts a bright blue cytoplasmic stain (Friedrich and Soriano, 1991). Recipient mice were strain-matched 1 month old female B6,129SF1 or C57/BL6 wild-type mice. All mice were purchased from Jackson Labs (Bar Harbor, ME). Animal studies were conducted according to protocols approved by the Boston University animal use committee and adhered strictly to NIH guidelines for the use and care of experimental animals.

Bone marrow cell harvest

Whole marrow was harvested by flushing femurs, tibiae, and humeri with ice cold Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY). Marrow cells were plated in plastic dishes at 10^6 cells/cm² and re-fed every 2-3 days (DMEM with 10% FBS and 1% penicillin-streptomycin). Subconfluent plastic-adherent cultured cells were harvested at 1 week by trypsinization (0.25% trypsin/1 mM EDTA) for 5 minutes at 37°C followed by gentle scraping. Cells were collected by centrifugation (800 rpm) and washed with PBS.

Bleomycin-induced lung injury followed by bone marrow cell injection

Recipient mice were anesthetized and treated with intratracheal bleomycin (0.075 Units bleomycin in 0.1 ml PBS) or PBS alone. Plastic-adherent cultured bone marrow cells ($1-2 \times 10^6$ in 0.2 ml PBS) were injected into the tail vein 5 days later. At 1, 2.5, 5, 14 or 30 days after marrow cell injection, mice were sacrificed by cervical dislocation followed by transection of the abdominal aorta.

Histological analysis of recipient lungs

Lungs were fixed for 15 minutes by instilling 0.25% glutaraldehyde in PBS (pH 7.4, 22°C) through a tracheal catheter. The fixative was removed before PBS lavage. X-gal solution (0.1% X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂ in PBS, pH 7.4) was instilled. After tracheal ligation, harvested lungs were incubated in X-gal solution overnight, washed with PBS, and fixed in 4% paraformaldehyde (Polysciences, Warrington, PA.) Intact lungs were screened under a dissecting microscope to localize engrafted blue-stained donor cells. Blue "dots" representing clusters of stained cells were microdissected, and embedded in paraffin or plastic (methyl methacrylate) and sectioned by standard methods. In addition, 20 random lung paraffin sections from the right lung of each recipient were processed for analysis.

Type I pneumocyte cell markers in engrafted cells

After sections containing *lacZ*-expressing cells were photographed, the coverslips were removed and the sections were processed for immunostaining or lectin binding. Before staining, sections were treated with hydrogen peroxide in methanol (0.3%, 15 minutes, 22°C) to quench endogenous peroxidases. For lectin binding, sections were incubated with 1% rabbit serum in PBS for 15 minutes, washed with PBS, and exposed to biotinylated *Lycopersicon esculentum* lectin (2 µg/ml; Vector Laboratories, Burlingame, CA) for 30 minutes. HRP-labeled avidin (5 µg/ml; Vector Laboratories) was applied for 30 minutes followed by the chromogen diaminobenzidine. The slides were counter-stained with Nuclear Fast Red. For T1α immunohistochemistry, antigen retrieval was performed by heating sections to 90°C in a citric acid buffer (antigen retrieval solution; Vector Laboratories) for 20 minutes, slowly cooling to room temperature, prior to quenching. Sections were blocked with 1% goat serum in PBS (60 minutes), incubated overnight (4°C) with a monoclonal hamster anti-mouse T1α antibody (Developmental Studies Hybridoma Bank, University of Iowa, Hybridoma #8.1.1, www.uiowa.edu/~dshbwww/, courtesy of Dr Andrew Farr) (Farr et al., 1992), and treated with HRP-labeled goat anti-hamster IgG (ICN, Costa Mesa, CA) for 30 minutes at room temperature. Signals were amplified using tyramide (TSA-Biotin System, NEN Life Science Products, Boston, MA) according to the manufacturer's protocol before exposure to diaminobenzidine (6.5 minutes). Cell proliferation was assessed immunohistochemically by staining for proliferating cell nuclear antigen (PCNA), using an anti-PCNA antibody according to manufacturer instructions (PCNA Staining Kit, Zymed Laboratories, San Francisco, CA).

Polymerase chain reaction (PCR) analyses

PCR analysis for the *lacZ*/neomycin resistance gene (*lacZ*/neo) (Friedrich and Soriano, 1991) was performed on DNA extracted from recipient tissue using primers that span the fusion gene (forward primer: CCGCATCCCCCTGCTGTCCCGTGCA, reverse primer: CTCCCCCAACCCCTGTCTGCTGT; 94°C 30 seconds, 53°C 60 seconds, 72°C 60 seconds, 35 cycles). The identity of the 140 base pair PCR product was confirmed by sequencing. To assess fidelity of PCR, the mouse surfactant protein C gene (*SP-C*) was also amplified in each sample. Lung tissue from the donor B6,129GtRosa 26 mouse strain served as positive control and lung tissue from a wild-type recipient B6,129SF1 served as negative control.

Immunohistochemical staining of bone marrow cultures and whole bone

Bone marrow obtained as above was plated on plastic chambered slides (3.5×10^5 cells/well) (Lab-Tek 8 well chambered Permax Slides, Naperville, IL,) and cultured as above. After 1 week, subconfluent adherent cultured cells were fixed with ice-cold 5% acetic acid in ethanol. Using the above staining methods, cells were immunostained for T1α protein expression. Whole murine femur was fixed in 4% paraformaldehyde, decalcified by immersion in Immunocal (Decal Chemical, Congers, NY) for 2 weeks before washing with deionized water, embedding in paraffin, sectioning and immunostaining for T1α.

Expression of lung epithelial mRNAs in bone marrow cells

10 µg of total RNA, isolated from fresh marrow aspirates and marrow cultured on plastic for 1 week was reverse transcribed (Promega Reverse Transcription System, Madison WI) before PCR amplification of T1α, aquaporin-5 (AQP-5), and surfactant protein-C (SP-C) cDNAs (forward primers: for T1α TGTGGACCGTGCC-AGTGTGTTT, for AQP-5 CCTTATCCATTGGCTTGTCGG, for SP-C ATTACTCGGCAGGTCCAGGAGCCA; reverse primers: for T1α TCATCTTCTCCACAGGAAGAGGA, for AQP-5 TCTGAG-CTGTGGCAGTCGTTT, for SP-C AGATATAGTAGAGTGTTAG-CTCTCC; 94°C 30 seconds, 55°C 60 seconds, 72°C 60 seconds, 30 cycles). Using identical amplification conditions, nested PCR was

subsequently performed with a second set of primers (forward primers for T1 α for AAGGCACCTCTGGTACCAACG, for AQP-5 TCTTCTGGGTAGGACCGATCG, for SP-C TTGTGGTGGTGGTCCTCGTT; reverse primers for T1 α ACAATGAAGATCCCTCCGACG, for AQP-5 GAGAGGTGCTCCAACTCTTCGT, for SP-C AGG-TCTCTCCCGGAAGAATC). The identity of bands was confirmed by size estimation and restriction mapping.

Statistical analysis

A recipient animal was defined as showing lung engraftment of donor-derived cells if at least one *lacZ*-expressing cell was found by X-gal staining. An engrafted cell cluster was arbitrarily defined as more than 3 engrafted cells seen in one high power field by X-gal staining. The proportion of animals in each group (bleomycin exposed versus PBS exposed) showing lung engraftment or showing cluster pattern engraftment was assessed by performing a Fisher's exact test. $P < 0.05$ was taken as a statistically significant difference.

RESULTS

Injected bone marrow-derived cells engraft in recipient lungs

X-gal-stained cells were readily apparent in bleomycin-treated lungs at 30 days after injection of adherent *lacZ*-positive cells. The engrafted cell clusters appeared as blue dots of variable size in the parenchyma of recipient lungs (Fig. 1A). PCR analysis confirmed the presence of the *lacZ/neo* fusion gene (Friedrich and Soriano, 1991) in the microdissected blue clusters (Fig. 1B). The identity of the expected 140 bp *lacZ/neo* PCR product was validated by DNA sequencing.

Assessment of engraftment in other organs

To assess whether donor cell seeding of recipient marrow was associated with lung engraftment, we analyzed recipient bone marrow by PCR and by X-gal histochemistry. We found no evidence of bone marrow engraftment in any of the mice with lung engraftment 2 or 4 weeks after injection. In some animals, donor cells were observed in recipient spleens suggesting that the injected marrow cells did circulate beyond the initially encountered lung capillary bed. In contrast, we did not observe engraftment in recipient livers or hearts.

Engraftment as type I pneumocytes

To examine the cell type of the lung-engrafted *lacZ*-positive cells, we performed a microscopic analysis of paraffin-embedded recipient lungs that contained blue X-gal-stained clusters (Fig. 2). These studies revealed clusters of flat blue lines along the alveolar surface. This morphology matches the normal location, size, and orientation of type I pneumocytes of the alveolar epithelium (Fig. 2A,B). For better cellular definition, blue dots from injured recipient lungs were also embedded in plastic. Analysis of plastic sections (1–2 μ m) confirmed that the *lacZ*-labeled donor cells had become flattened cells that lined alveoli, contained ovoid nuclei that bulged slightly into the alveolar lumen, and were located adjacent to type II cells. These are features that uniquely define type I pneumocytes of the alveolar epithelium (Dobbs et

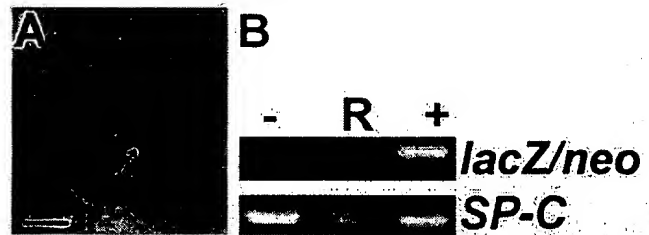


Fig. 1. Confirmation of donor origin of lung-engrafted X-gal-positive cell cluster. Bone marrow cells from a donor mouse transgenic for a *lacZ/neo* resistance fusion gene (*lacZ/neo*) were injected into a wild-type recipient with bleomycin-injured lungs. Recipient lungs were harvested at one month and stained with X-gal to identify injected cells. (A) Donor-derived *lacZ*-positive cells can be seen under a dissecting microscope as a diffuse area of blue staining. (B) The blue cell cluster seen in A was microdissected for DNA extraction and PCR for the *lacZ/neo* fusion gene. Donor lung DNA served as a positive control whereas wild-type lung DNA served as a negative control. To confirm fidelity of PCR, the mouse surfactant C gene (SP-C) was also amplified in each sample. (–), Negative control; R, recipient lung; +, positive control. Bar, 100 μ m.

al., 1988) (Fig. 2D). In areas where engrafted type I cells abutted type II cells, the blue X-gal staining of the type I cell cytoplasm abruptly ceased at the type I-type II cell interface.

A summary of our findings for animals sacrificed 30 days after cell injection is shown in Fig. 3. Bleomycin-induced lung injury affected both the frequency and the pattern of lung engraftment in recipient mice. There was a statistically significant difference in the proportion of mice showing lung engraftment in the bleomycin-exposed group (4 of 4 mice) compared to the PBS-exposed group (2 of 9 mice; $P = 0.02$). We

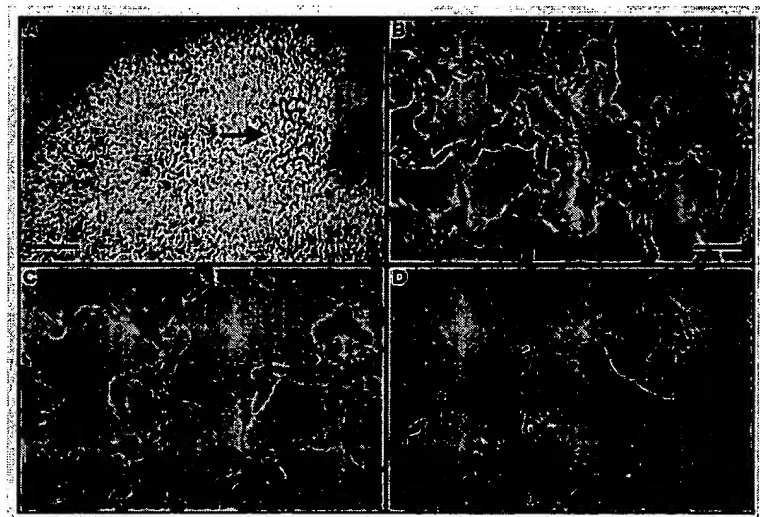


Fig. 2. Paraffin and plastic sections of X-gal-stained mouse lungs 30 days after cell injection. (A) Low magnification (40 \times) and (B) high magnification (1000 \times) views of a representative recipient lung showing engrafted donor-derived cells as thin, flat blue cells consistent with a type I pneumocyte morphology. (C) For comparison, an X-gal-stained negative control lung (*lacZ*-negative marrow injected into a *lacZ*-negative recipient) shows no blue cells. (D) A thin plastic section (2 μ m) of recipient mouse lung confirms the type I pneumocyte morphology of engrafted donor-derived cells. Sections are counter-stained with Nuclear Fast Red. Bars, 200 μ m (A); 30 μ m (B–D).

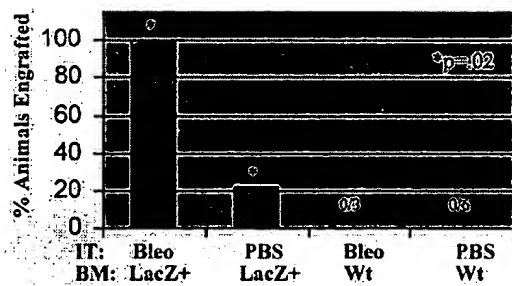


Fig. 3. Summary of lung engraftment in animals sacrificed 30 days after injection of *lacZ*-positive or wild-type (Wt) cultured plastic-adherent bone marrow cells (BM). Injections were performed 5 days after intra-tracheal (IT) bleomycin (Bleo) or control vehicle (PBS) exposure. There was a statistically significant increase in the proportion of mice showing lung engraftment following bleomycin treatment ($*P<0.02$). No animals that received wild-type (non-*lacZ*) cells displayed positive staining (0/9).

also found a difference in engraftment patterns between the groups. PBS-exposed mice showed single engrafted cells located either at or near the pleural surface, whereas bleomycin-exposed mice showed engraftment as peripheral clusters of numerous type I pneumocytes in 3 of 4 engrafted mice ($P=0.014$). Bleomycin-exposed recipients showed 2-6 clusters/lung. All animals that received wild-type, non-*lacZ*-expressing bone marrow injections did not stain for X-gal.

Assessment of type I cell molecular markers by engrafted cells

We performed lectin histochemistry and T1 α immunohistochemistry to assess expression of type I cell-specific markers on engrafted *lacZ*-expressing cells. We first examined lung engrafted cells for binding to the *Lycopersicon esculentum* lectin. In lung tissue, this lectin binds to the surface of type I pneumocytes, but does not bind to type II cells or lung endothelium (Bankston et al., 1991). A representative lung section containing engrafted cells subjected to lectin staining, is shown in Fig. 4. We found that all lung engrafted X-gal-stained cells bound *Lycopersicon esculentum* lectin.

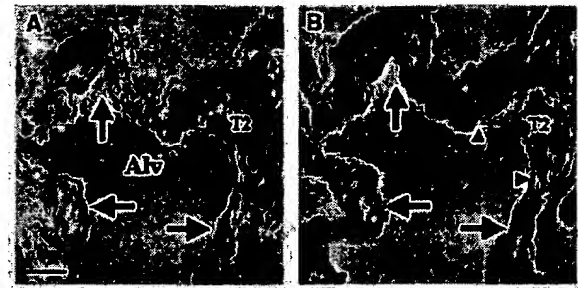


Fig. 4. Co localization of *Lycopersicon esculentum* lectin and X-gal staining of engrafted type I pneumocytes. (A) Type I pneumocytes (arrows) lining an alveolus (Alv) stain blue indicating their origin from *lacZ*-expressing bone marrow cells. No X-gal staining was found in the adjacent type II cell (T2). (B) This same section after binding to *L. esculentum* lectin shows that the brown lectin staining co-localizes with the blue cells. The lectin staining stops abruptly at the junction of positively stained type I cells and the negatively stained type II cell. Bar, 7 μ m.

We subsequently assessed the expression of T1 α in the engrafting cell population in recipient lungs. In normal rodent lung, this protein is expressed only in type I cells and is targeted to the apical surface (Cao et al., 2000; Danto et al., 1992; Ramirez et al., 1997; Rishi et al., 1995; Williams et al., 1996). The hamster anti-mouse T1 α monoclonal antibody employed in this analysis has staining characteristics identical to T1 α antibodies used on rat tissue, and as expected, specifically identifies a single protein of ~38 kDa in homogenates of mouse lung (Y. X. Cao, M. I. Ramirez and M. C. W., unpublished). Immunostaining of *lacZ*-positive donor lungs (Fig. 5A-C) demonstrates that both *lacZ* and T1 α markers can be identified on a single section and illustrates the typical linear staining pattern for a type I pneumocyte marker (Dobbs et al., 1988; Williams et al., 1996). Panels A-C establish the antigen specificity of this antibody after X-gal staining and re-confirm that the T1 α protein is not expressed in type II pneumocytes (Fig. 5A), endothelium (Fig. 5B), and alveolar macrophages (Fig. 5C). We immunostained recipient lungs (Fig. 5D,E) and found that the blue engrafted donor cells

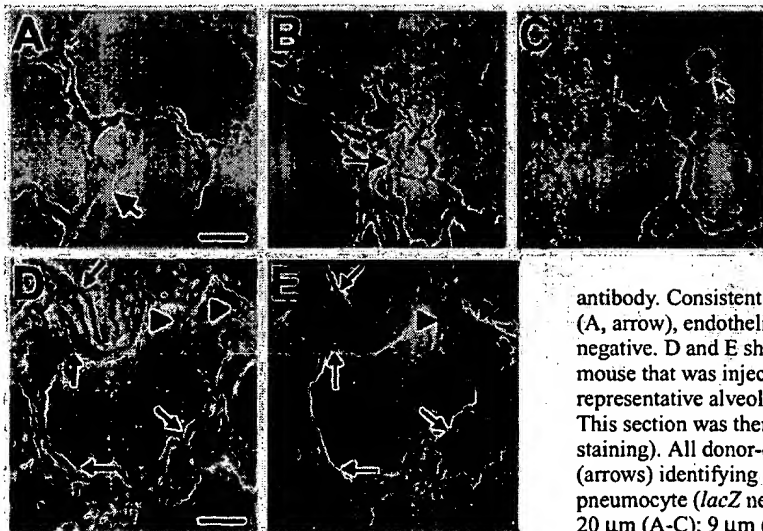


Fig. 5. Double labeling for T1 α (brown) and *lacZ* expression (blue) in donor (A-C) and recipient (D,E) lungs. A-C show donor lungs from *lacZ*-positive transgenic mice that constitutively express *lacZ* in all cells. These panels demonstrate that: (1) in the *lacZ* mouse X-gal histochemistry stains all *lacZ*-expressing lung cells blue; (2) immunohistochemical brown staining of a type I pneumocyte membrane protein produces a characteristic linear staining pattern; (3) X-gal staining does not alter the specificity of the T1 α

antibody. Consistent with the specificity of this type I cell marker, type II cells (A, arrow), endothelium (B, arrow), and macrophages (C, arrow) are T1 α negative. D and E show sections from an experimental wild-type recipient mouse that was injected with donor *lacZ*-positive marrow-derived cells. A representative alveolus contains blue *lacZ*-positive flattened engrafted cells (D). This section was then re-stained with the T1 α monoclonal antibody (E; brown staining). All donor-derived blue cells are also positive for T1 α protein staining (arrows) identifying them as type I pneumocytes. An endogenous type I pneumocyte (*lacZ* negative, T1 α positive) is also indicated (arrowheads). Bars, 20 μ m (A-C); 9 μ m (D,E).

express T1 α , further evidence that these marrow-derived cells adopt molecular phenotypic features of type I pneumocytes.

Lung engraftment at earlier time points

At 5 and 14 days after injection into bleomycin-treated mice, engrafted cell clusters could be observed; all engrafted cells had a type I cell morphology (Fig. 6). No *lacZ*-expressing type II pneumocytes were observed at any time point.

To further evaluate the possibility that donor cells engraft first as type II cells and later acquire the type I cell phenotype, bleomycin-injured mice were also sacrificed at 1 and 2.5 days after injection. At these time points, donor-derived cells could be detected both in the pulmonary vasculature (Fig. 7A), and in the injured recipient lung parenchyma. Within the parenchyma, clumps of 1-3 donor cells were found along the alveolar epithelial surface (Fig. 7B-D). These cells appeared elongated with attenuated cytoplasmic extensions, and none possessed the location, shape, or lamellar bodies characteristic of type II pneumocytes when examined by light and phase-contrast microscopy (Fig. 7E). For comparison, no positive X-gal-stained cells were observed at either time point in bleomycin-injured wild-type recipients injected with wild-type (non-*lacZ* expressing) cultured marrow.

PCNA Immunostaining of lung engrafted cells

We used PCNA immunohistochemistry (Waseem and Lane, 1990) to assess for evidence of cell proliferation in lung engrafted cells from bleomycin-exposed recipients 1, 2.5 and 5 days after cultured marrow injection (Fig. 7F). We found no positive PCNA staining in engrafted cells. In addition, no mitotic figures were seen in any lung engrafted cell at any time.

Expression of type I pneumocyte markers in bone marrow cultures

Plastic-adherent cultures of donor bone marrow cells display three discernible morphologic subtypes (Fig. 8A). These include small rounded cells, small stellate cells and large polygonal fibroblast-like cells (Phinney et al., 1999; Wang and Wolf, 1990). To examine whether any of these cell types express type I pneumocyte markers during culturing, we performed T1 α immunohistochemistry. Approximately 10% of cells are T1 α positive after 1 week of culturing on plastic (Fig. 8B). T1 α expression appears to be limited to cells with the morphology of the fibroblast-like cell sub-population, as shown by their polygonal shape (Fig. 8C). Within an aggregate of fibroblast-like cells, T1 α expression is variable, as not all cells express this marker at detectable levels (Fig. 8D). For comparison, we immunostained cultures for a pan-endothelial marker (Leppink et al., 1989; Phinney et al., 1999). Consistent with reported work, this antigen is expressed in the small rounded cells that likely represent endothelial precursors; this sub-population was T1 α negative (data not shown).

Nested RT-PCR analyses on mRNA derived from plastic-adherent bone marrow cell cultures show that T1 α mRNA is expressed (Fig. 9). In addition, T1 α

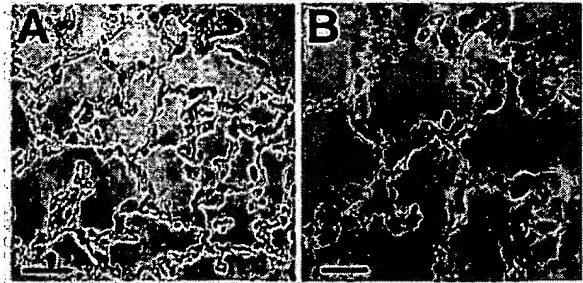


Fig. 6. Paraffin section of recipient injured lung 5 days after injection of *lacZ*-positive marrow-derived cells. Linear blue staining indicates engraftment of *lacZ*-positive donor marrow-derived cells as type I pneumocytes as in the 30 day recipients. No blue type II pneumocytes were observed. Bars, 40 μ m (A); 20 μ m (B).

mRNA is expressed in fresh bone marrow aspirates (Fig. 9). Aquaporin-5, another type I cell-specific marker (Funaki et al., 1998), is also expressed in both marrow cell preparations (Fig. 9). No signal was observed in either cell sample for surfactant protein C (SP-C) mRNA, a marker specific for alveolar type II cells (Fisher et al., 1989).

To further examine expression of T1 α in bone marrow cells, we immunostained a decalcified murine femur embedded in paraffin. We found that a population of ovoid cells located in the bone immediately subjacent to the marrow cavity was T1 α positive (Fig. 10). There were no cells within the marrow cavity

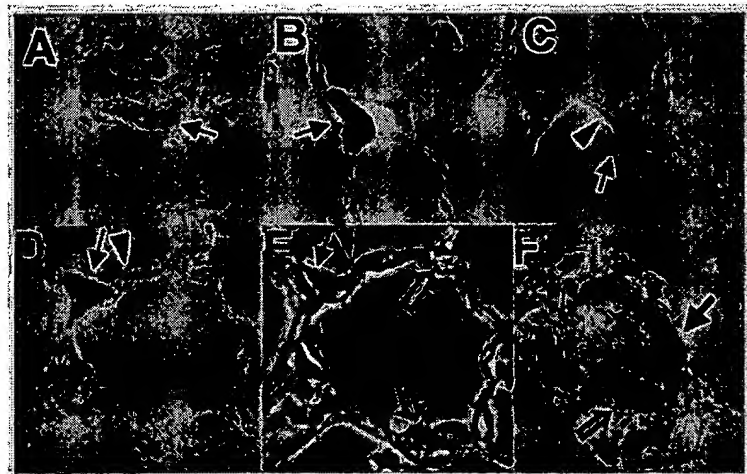


Fig. 7. Donor bone-marrow-derived cells (blue) in recipient bleomycin-injured lungs 1 and 2.5 days after injection. (A,B) Ovoid donor cells against a pulmonary blood vessel wall (arrow; A) and in the injured lung parenchyma (arrow; B) 1 day after injection. (C,D) By 2.5 days, donor-derived cells can be found in the lung parenchyma as elongated cells (arrows) with attenuated cytoplasmic extensions (arrowheads). As shown in A-D, engrafted cells do not exhibit the location and shape of type II cells. (E) Phase microscopy of D demonstrating that engrafted cells do not contain lamellar bodies. For comparison, two neighboring endogenous type II cells (*lacZ* negative; open arrows) with characteristic lamellar bodies are shown. (F) PCNA immunostained lung section with a blue donor-derived cell 2.5 days after injection. This nucleus (arrow) does not stain for proliferating cell nuclear antigen (PCNA, brown). In contrast, a neighboring *lacZ*-negative cell stains positively for PCNA (open arrow; nuclei counter stained purple with Haematoxylin).

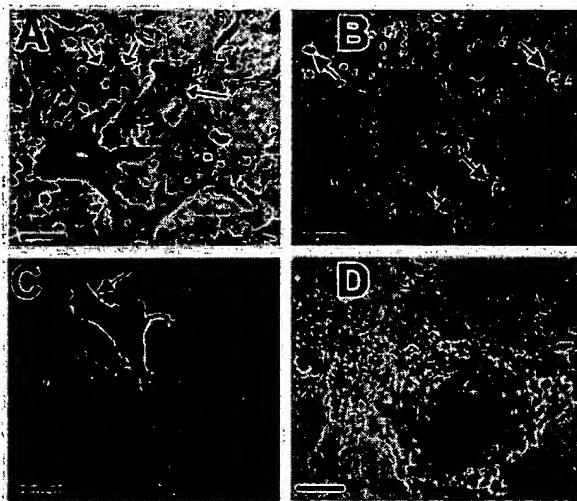


Fig. 8. Histological analyses of adherent bone marrow cultures. (A) Three cell morphologies are discernible: larger polygonal fibroblast-like cells (black arrow), smaller stellate myeloid cells (red arrow), and small round endothelial-like cells (purple arrow). (B-D) Immunostaining of cultures with anti-mouse T1 α antibody (brown staining; B, arrows). (C) At higher magnification, the T1 α -positive cell demonstrates fibroblast-like morphology. (D) Staining within a cell colony is heterogeneous. Bars, 20 μ m (A); 200 μ m (B); 15 μ m (C); 25 μ m (D).

that stained positive for T1 α . These findings are in agreement with previous work showing that T1 α is expressed in a subset of osteoblasts at the time of their encapsulation in the bony matrix (Wetterwald et al., 1996).

DISCUSSION

The present study is the second description of a bone-marrow derived cell capable of forming lung alveolar epithelium (Krause et al., 2001), and demonstrates for the first time that cultured bone marrow cells can serve as type I pneumocyte precursors. By injecting *lacZ*-positive plastic-adherent cultured bone marrow cells into recipient mice, we demonstrated

engraftment of marrow-derived cells in recipient lungs as cells possessing both the morphologic and molecular phenotype of type I pneumocytes. While these are unique findings, they are consistent with recent work showing that bone marrow-derived cells can serve as progenitors for differentiated parenchymal cells of organs such as the brain (Kopen et al., 1999; Prockop et al., 2000; Woodbury et al., 2000), liver (Lagasse et al., 2000; Petersen et al., 1999) and epithelia of the gastrointestinal tract and lung (Krause et al., 2001).

The distinct cellular localization, morphology and molecular features of the engrafted cells indicate that the engrafted cells express the type I pneumocyte phenotype, and the PCR data confirm that positive blue X-gal staining arises from locally expressed *E. coli*-derived *lacZ*. We considered the possibility that the observed staining patterns resulted from the diffusion of the X-gal reaction product from another cellular source. We did not detect, however, positive staining in any other cell type within the alveolus. This is consistent with previous work showing that cell-specific expression of *lacZ* in the alveolar epithelium remains localized and does not leak into neighboring cells (Strayer et al., 1998). Since no staining was observed in lungs from animals that received wild-type bone marrow cells that do not carry the *lacZ* transgene, positive blue staining cannot be attributed to endogenous murine galactosidase expression. In keeping with these results, previous work showed that endogenous galactosidase activity is only observed at acidic pH (Dimri et al., 1995; Severino et al., 2000; Weiss et al., 1997; Weiss et al., 1999); notably all our experiments were performed at pH 7.4.

We found that animals exposed to bleomycin are more likely to show lung engraftment of bone marrow-derived cells than PBS-treated animals. The oxidative damage produced by bleomycin leads to both alveolar endothelial and epithelial injury, inflammatory exudation, and fibroblast recruitment which in turn lead to enhanced alveolar cell turnover, increased permeability, and localized release of effector substances (Adamson and Bowden, 1979). These events could all conceivably contribute to the engraftment process. In our studies, marrow cell injection coincided with the point at which bleomycin-induced edema and necrosis of alveolar endothelial cells has already occurred, and type I pneumocyte injury is just beginning.

We believe our findings differ from previous work on lung

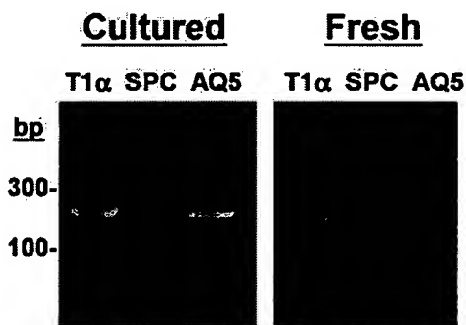


Fig. 9. Nested RT-PCR analysis for lung epithelial markers in plastic-adherent cultured marrow cells (7 days) and fresh bone marrow. Positive expression of T1 α and aquaporin-5 (AQ5) was noted under both conditions. Surfactant protein-C (SPC) was not expressed.

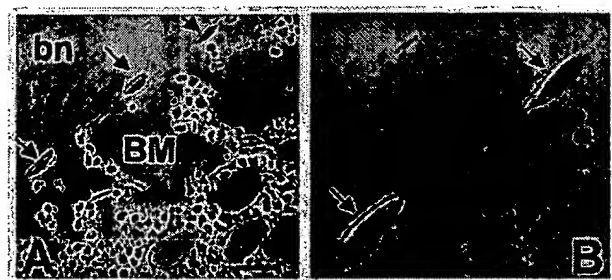


Fig. 10. Immunostaining for T1 α in whole decalcified mouse bone marrow. (A) Low power and (B) high power view of elongated T1 α -positive cells (arrows) on interior surface of bone, facing the marrow cavity. Cells within the marrow cavity were negative. bn, bone; BM, bone marrow cavity; Fast Nuclear Red counter stain; Bar, 25 μ m.

engraftment by Krause et al., because of significant differences in experimental protocols. Krause et al., demonstrated that an uncultured bone marrow-derived stem cell was able to achieve multi-lineage engraftment in irradiated recipient mice, including lung engraftment as bronchial epithelium and type II pneumocytes, 11 months after bone marrow transplantation (Krause et al., 2001). In contrast, we observed lung engraftment of marrow-derived cells only as type I pneumocytes and did not find detectable marrow engraftment. Our use of recipients with intact bone marrow, induction of lung injury with bleomycin, and selective expansion of plastic-adherent cells could all conceivably account for our unique results. These cells, expanded on plastic, constitute only a small percentage of normal marrow. In addition, their functions and phenotypes may be altered by culturing. Finally, whether the type of marrow-derived cell used by Krause et al., was present in our cultures is not known. Our results, therefore, are consistent with the possibility that marrow-derived cells can engraft as other lung phenotypes under different experimental conditions.

No donor-derived type II pneumocytes were detected at any time, but we cannot completely exclude the possibility that engrafted type II cells were present prior to the first time point we studied (24 hours). If donor-derived type II cells were present, they could serve as a transient amplifying population that subsequently gives rise to type I cells, as is known to be the case in lung injuries (Adamson and Bowden, 1974). This possibility seems unlikely, however, since type II cells are a self-renewing local stem cell population. Residual type II cells expressing the genomic *lacZ* marker should be present if type II cell engraftment had initially occurred. Indeed, previous work studying tritiated thymidine-labeled type II pneumocytes in vivo showed that type II daughter cells remain labeled even after one of the progeny has differentiated into a type I cell (Adamson and Bowden, 1974), and Krause et al.'s report achieving marrow engraftment as type II cells showed these cells were detectable as early as 5 days and as late as 11 months after injection (Krause et al., 2001). It is also important to note that the type II cells in our X-gal-stained donor lungs constitutively express *lacZ* (Fig. 5A), indicating that any engrafted type II cell should similarly be detectable by X-gal staining.

We identified a clustered pattern of cell engraftment in injured lungs beginning 5 days after injection. These clusters could theoretically arise in several ways: a single marrow-derived cell might engraft in lung tissue and then clonally expand into a cell cluster, multiple individual cells might engraft separately in the same location, or a clump of injected cells could lodge in a lung vessel and engraft en masse. Our data do not yet distinguish between these possibilities. However, in preliminary work, we found no cell clusters present before 5 days after injection, and no cell clumps larger than 3 cells lodged in any pulmonary vessel. Furthermore, we found no evidence of proliferating engrafted cells studied by PCNA staining at 1, 2.5, and 5 days after injection, suggesting that clonal expansion may not be responsible for cluster formation. This clustered pattern of engraftment has also been observed in liver when marrow-derived cells engraft as differentiated hepatocytes during metabolically induced injury (Lagasse et al., 2000). Engraftment as cell clusters is therefore not unique to the lung.

These studies demonstrate that bone marrow cells are capable of expressing markers specific for type I cells, including T1 α and aquaporin-5, and the RT-PCR data indicate the presence of T1 α -expressing cells within aspirates prior to culturing. In cultured cells, we found T1 α -expressing cells within colonies of cells with a polygonal morphology. This cell sub-type is believed to serve as pluripotent stem cells for muscle, bone, and fat (Phinney et al., 1999; Pittenger et al., 1999; Prockop, 1997). Colonies of these cells are believed to evolve in culture as a result of clonal expansion from a single adherent marrow cell (Bianco and Robey, 2000; Wang and Wolf, 1990). By immunostaining whole de-calcified bone, we found, as has been reported (Wetterwald et al., 1996), a T1 α -positive long thin cell on the interior aspect of the bone facing the marrow cavity. These cells are believed to be osteoblasts or pre-osteocytes (Wetterwald et al., 1996). We do not yet know whether this cell was present in our original marrow aspirates and subsequently served as a precursor to the population of T1 α -positive cultured cells.

The functional relationship between expression of type I pneumocyte markers by adherent bone marrow cells and engraftment potential in the alveolar epithelium is uncertain at this time. Our observations raise intriguing questions regarding the relative role of lung derived cues in establishing a lung epithelial phenotype. It is possible that cultured bone marrow-derived cells display certain features of lung differentiation prior to engraftment. It is also possible that multipotent marrow cells lose their engraftment potential as they express differentiation markers, such as T1 α during culturing.

In conclusion, we believe that these studies provide a rationale for the eventual use of bone marrow progenitors in the treatment of conditions in which failure of the intrinsic regenerative capacity of damaged organs is a central pathological feature. In the lung, such an approach may be relevant for conditions associated with acute and extensive injury to the gas exchange surface.

We thank Dr Jerome Brody for thoughtful guidance and Anne Hinds for technical assistance with histology specimens. This work was supported by NIH grants P01HL47049 and U19AI41994.

REFERENCES

- Adamson, I. Y. R. and Bowden, D. H. (1974). The type 2 cell as progenitor of alveolar epithelial regeneration. *Lab. Invest.* 30, 35-42.
- Adamson, I. Y. R. and Bowden, D. H. (1979). Bleomycin-induced injury and metaplasia of alveolar type 2 cells. *Am. J. Pathol.* 96, 531-544.
- Alison, M. R., Poulsom, R., Jeffrey, R., Dhillon, A. P., Quaglia, A., Jacob, J., Novelli, M., Prentice, G., Williamson, J. and Wright, N. A. (2000). Hepatocytes from non-hepatic adult stem cells. *Nature* 406, 257.
- Bankston, P. W., Porter, G. A. and Millic, A. J. P. G. E. (1991). Differential and specific labeling of epithelial and vascular endothelial cells of the rat lung by Lycopersicon esculentum and Griffonia simplicifolia I lectins. *Eur. J. Cell Biol.* 54, 187-195.
- Bianco, P. and Robey, P. G. (2000). Marrow stromal stem cells. *J. Clin. Invest.* 105, 1663-1668.
- Cao, Y. X., Jean, J. C. and Williams, M. C. (2000). Cytosine methylation of an Sp1 site contributes to organ-specific and cell-specific regulation of expression of the lung epithelial gene T1 α . *Biochem. J.* 350, 883-890.
- Conget, P. A. and Minguell, J. J. (1999). Phenotypal and functional properties of human bone marrow mesenchymal progenitor cells. *J. Cell. Physiol.* 181, 67-73.
- Danto, S. I., Zabski, S. M. and Crandall, E. D. (1992). Reactivity of alveolar

- epithelial cells in primary culture with type I cell monoclonal antibodies. *Am. J. Respir. Cell. Mol. Biol.* 6, 296-306.
- Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O. et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* 92, 9363-9367.
- Ding, L., Lu, S., Batchu, R., III, R. S. and Munshi, N. (1999). Bone marrow stromal cells as a vehicle for gene transfer. *Gene Ther.* 6, 1611-1616.
- Dobbs, L. G., Williams, M. C. and Gonzalez, R. (1988). Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated type II cells. *Biochim. Biophys. Acta* 970, 146-156.
- Eglitis, M. A. and Mezey, E. (1997). Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc. Natl. Acad. Sci. USA* 94, 4080-4085.
- Farr, A. G., Berry, M. L., Kim, A., Nelson, A. J., Welch, M. P. and Aruffo, A. (1992). Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. *J. Exp. Med.* 176, 1477-1482.
- Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors [see comments] [published erratum appears in *Science* 1998 Aug 14;281 (5379):923]. *Science* 279, 1528-1530.
- Fisher, J. H., Shannon, J. M., Hofmann, T. and Mason, R. J. (1989). Nucleotide and deduced amino acid sequence of the hydrophobic surfactant protein SP-C from rat: expression in alveolar type II cells and homology with SP-C from other species. *Biochim. Biophys. Acta* 995, 225-230.
- Friedrich, G. and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* 5, 1513-1523.
- Funaki, H., Yamamoto, T., Koyama, Y., Kondo, D., Yaoita, E., Kawasaki, K., Kobayashi, H., Sawaguchi, S., Abe, H. and Kihara, I. (1998). Localization and expression of AQP5 in cornea, serous salivary glands, and pulmonary epithelial cells. *Am. J. Physiol.* 275, C1151-C1157.
- Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M. and Mulligan, R. C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401, 390-394.
- Hou, Z., Nguyen, Q., Frenkel, B., Nilsson, S. K., Milne, M., Van Wijnen, A. J., Stein, J. L., Quesenberry, P., Lian, J. B. and Stein, G. S. (1999). Osteoblast-specific gene expression after transplantation of marrow cells: implications for skeletal gene therapy. *Proc. Natl. Acad. Sci. USA* 96, 7294-7299.
- Kopen, G. C., Prockop, D. J. and Phinney, D. G. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA* 96, 10711-10716.
- Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Sharkis, S. J. (2001). Multi-organ, multilineage engraftment by a single bone marrow-derived stem cell. *Cell* 105, 369-377.
- Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, J. L. and Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat. Med.* 6, 1229-1234.
- Lemischka, I. (1999). The power of stem cells reconsidered? *Proc. Natl. Acad. Sci. USA* 96, 14193-14195.
- Leppink, D. M., Bishop, D. K., Sedmak, D. D., Henry, M. L., Ferguson, R. M., Streeter, P. R., Butcher, E. C. and Orosz, C. G. (1989). Inducible expression of an endothelial cell antigen on murine myocardial vasculature in association with interstitial cellular infiltration. *Transplantation* 48, 874-877.
- Liechty, K. W., MacKenzie, T. C., Shaaban, A. F., Radu, A., Moseley, A. M., Deans, R., Marshak, D. R. and Flake, A. W. (2000). Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. *Nat. Med.* 6, 1282-1286.
- Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O. and Pittenger, M. F. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* 4, 415-428.
- Nilsson, S. K., Dooner, M. S., Weier, H. U., Frenkel, B., Lian, J. B., Stein, G. S. and Quesenberry, P. J. (1999). Cells capable of bone production engraft from whole bone marrow transplants in nonablated mice. *J. Exp. Med.* 189, 729-734.
- Ono, K., Takii, T., Onozaki, K., Ikawa, M., Okabe, M. and Sawada, M. (1999). Migration of exogenous immature hematopoietic cells into adult mouse brain parenchyma under GFP-expressing bone marrow chimera. *Biochem. Biophys. Res. Commun.* 262, 610-614.
- Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M., Leri, A. and Anversa, P. (2001). Bone marrow cells regenerate infarcted myocardium. *Nature* 410, 701-705.
- Pereira, R. F., Halford, K. W., O'Hara, M. D., Leeper, D. B., Sokolov, B. P., Pollard, M. D., Bagasra, O. and Prockop, D. J. (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc. Natl. Acad. Sci. USA* 92, 4857-4861.
- Pereira, R. F., O'Hara, M. D., Laptev, A. V., Halford, K. W., Pollard, M. D., Class, R., Simon, D., Livezey, K. and Prockop, D. J. (1998). Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc. Natl. Acad. Sci. USA* 95, 1142-1147.
- Petersen, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S. and Goff, J. P. (1999). Bone marrow as a potential source of hepatic oval cells. *Science* 284, 1168-1170.
- Phinney, D. G., Kopen, G., Isaacson, R. L. and Prockop, D. J. (1999). Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J. Cell. Biochem.* 72, 570-585.
- Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143-147.
- Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 276, 71-74.
- Prockop, D. J., Azizi, S. A., Colter, D., Digirolo, C., Kopen, G. and Phinney, D. G. (2000). Potential use of stem cells from bone marrow to repair the extracellular matrix and the central nervous system. *Biochem. Soc. Trans.* 28, 341-345.
- Ramirez, M. I., Rishi, A. K., Cao, Y. X. and Williams, M. C. (1997). TGT3, thyroid transcription factor 1, and Sp1 elements regulate transcriptional activity of the 1.3-kilobase pair promoter of T1 α , a lung alveolar type I cell gene. *J. Biol. Chem.* 272, 26285-26294.
- Rishi, A. K., Joyce-Brady, M., Fisher, J., Dobbs, L. G., Floros, J., VanderSpek, J., Brody, J. S. and Williams, M. C. (1995). Cloning, characterization, and development expression of a rat lung alveolar type I cell gene in embryonic endodermal and neural derivatives. *Dev. Biol.* 167, 294-306.
- Severino, J., Allen, R. G., Balin, S., Balin, A. and Cristofalo, V. J. (2000). Is beta-galactosidase staining a marker of senescence in vitro and in vivo? *Exp. Cell Res.* 257, 162-171.
- Strayer, M. S., Guttentag, S. H. and Ballard, P. L. (1998). Targeting type II and Clara cells for adenovirus-mediated gene transfer using the surfactant protein B promoter. *Am. J. Respir. Cell Mol. Biol.* 18, 1-11.
- Wang, Q. R. and Wolf, N. S. (1990). Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. *Exp. Hematol.* 18, 355-359.
- Waseem, N. H. and Lane, D. P. (1990). Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J. Cell. Sci.* 96, 121-129.
- Weiss, D. J., Liggitt, D. and Clark, J. G. (1997). In situ histochemical detection of beta-galactosidase activity in lung: assessment of X-Gal reagent in distinguishing lacZ gene expression and endogenous beta-galactosidase activity. *Hum. Gene Ther.* 8, 1545-1554.
- Weiss, D. J., Liggitt, D. and Clark, J. G. (1999). Histochemical discrimination of endogenous mammalian beta-galactosidase activity from that resulting from lac-Z gene expression. *Histochem. J.* 31, 231-236.
- Wetterwald, A., Hoffstetter, W., Cecchini, M. G., Lanske, B., Wagner, C., Fleisch, H. and Atkinson, M. (1996). Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone* 18, 125-132.
- Williams, M. C., Cao, Y., Hinds, A., Rishi, A. K. and Wetterwald, A. (1996). T1 α protein is developmentally regulated and expressed by alveolar type I cells, choroid plexus, and ciliary epithelia of adult rats. *Am. J. Respir. Cell Mol. Biol.* 14, 577-585.
- Woodbury, D., Schwarz, E. J., Prockop, D. J. and Black, I. B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* 61, 364-370.